General Introduction

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Chronic obstructive pulmonary disease (COPD) is currently ranked as the fourth leading cause of death in the world, and is expected to be the third in 2020 (Mannino and Kiriz 2006). The disease is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response of the lungs and the airways to noxious particles or gases (GOLD 2015). Currently, none of the existing medications used to treat COPD have been shown to improve the long-term decline of lung function. Thus, COPD still represents a public health challenge with the urge for disease prevention and novel treatment options (GOLD 2015).

A Brief History of COPD

More than 300 years ago, the symptoms of COPD have first been noticed and recorded by physicians and various names have been used for their description (Petty 2006). In 1679, the Swiss physician Bonet referred to COPD as 'voluminous lungs' (Bonet 1679). In 1769, the Italian anatomist Giovanni Morgagni reported 19 cases of 'turgid' lungs (Morgagni 1769). During the 19th century, the British physician Charles Badham described bronchiolitis and chronic bronchitis as disabling disorders (Badham 1814), and the French physician René Laennec added emphysema as a component of COPD (Laennec 1829). However, it was not before 1959, during a gathering of medical professionals during the Ciba Guest Symposium, that the definition and diagnosis of COPD as we know it today was defined (Fletcher et al. 1959), whereas the term COPD was first used in 1965 by Dr. William Briscoe during the 9th Aspen Emphysema Conference (Briscoe and Nash 1965). Eleven years later, Dr. Charles Fletcher and his colleagues noticed that stopping tobacco smoking could help to slow down the progression of COPD, linking smoking to the development of COPD (Fletcher et al. 1977). Today, exposure to tobacco smoke is recognized as one of the leading risk factors for COPD.

Pathophysiology of COPD

COPD is characterized by chronic inflammation and structural changes of particularly the small airways and the lung parenchyma, which may underlie the progressive and irreversible airflow limitation. The inflammation is caused by infiltration of different inflammatory cells, particularly neutrophils and macrophages, into the lung, that may be involved in the development of emphysema and airway remodeling (O'Donnell et al. 2006). Pulmonary emphysema is the destruction of alveolar tissue. This may cause reduced oxygen uptake and loss of elastic recoil due to a reduced number of alveolar attachments. The loss of tethering results in collapse of the small airways, leading to airflow limitation and reduced gas exchange (McDonough et al. 2011). Emphysema is importantly attributed to increased

activity of proteolytic enzymes released by inflammatory cells (McDonough et al. 2011). Next to emphysema, airway remodeling is another important structural feature of COPD. Airway remodeling in COPD is characterized by peribronchial fibrosis and increased airway smooth muscle mass, thickening the airway wall, as well as mucus gland hypertrophy and goblet cell hyperplasia that are involved in mucus hypersecretion in these patients (Burgel and Martin 2010). Since the topic of tissue remodelling in COPD is not the main focus of this thesis, we refer the reader to excellent recent reviews on this particular topic (Postma and Timens 2006; Salazar and Herrera 2011; Chilosi et al. 2012; Dournes and Laurent 2012; Portillo and Morera 2012; Hirota and Martin 2013; Bidan et al. 2015).

Clinical diagnosis of COPD is vital, and should be considered for any patient who has COPD symptoms or a history of exposure to risk factors such as cigarette smoke (GOLD 2015). The most common diagnosis is spirometry. Spirometry is a test of pulmonary function which measures the forced expiratory volume in one second (FEV_1) and the forced vital capacity (FVC). The FEV_1 represents the volume of air that can be maximally breathed out in the *first second* of a breath, and FVC represents the volume of air that can be maximally breathed out in a single large breath (Kormos and Chick 2010). COPD is defined by a ratio of FEV_1/FVC < 0.7. The severity of COPD is divided in four different stages according to the FEV1 as % predicted (GOLD 2015). According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD), stage 1 or mild COPD is defined by an FEV1 > 80%, stage 2 or moderate COPD by an FEV_1 of 50-80%, stage 3 or severe COPD by an FEV_1 of 30-50% and very severe COPD by an FEV_1 < 30% (GOLD 2015). While FEV_1 and FVC have generally been accepted as the most important lung function parameters for the diagnosis of COPD, airway hyperresponsiveness (AHR) is also present in many of the patients (Tashkin et al. 1996; Postma and Kerstjens 1998; van den Berge et al. 2012). AHR is a common feature of asthma, another obstructive lung disease, and defined by an exaggerated obstructive response of the airways to a variety of pharmacological, chemical and physical stimuli, including histamine, methacholine, AMP, sulphur dioxide, fog and cold air (Hargreave et al. 1985; Postma and Kerstjens 1998; Meurs et al. 2008). In COPD, AHR represents an important characteristic, and is well described in an epidemiological and clinical context with rather consistent results (Vestbo and Hansen 2001; Hansen and Vestbo 2005; Scichilone et al. 2006; van den Berge et al. 2012). It has been reported that the AHR is associated with an accelerated decline in FEV_1 (Campbell et al. 1985; Postma et al. 1986; Tashkin et al. 1996), and could even be an independent predictor of mortality in COPD patients (Hospers et al. 2000). Moreover, there is a strong association between accumulation of lung neutrophils and AHR (van den Berge et al. 2012). Increases in the production of reactive oxygen species (ROS) and a number of potent pro-inflammatory cytokines are associated with its development (Postma et al. 1988; Chung 2001). Glucocorticosteroids are a class of corticosteroids, which can reduce inflammation via activation of glucocorticosteroid receptors (Rhen and Cidlowski 2005). Glucocorticosteroids

are used to treat diseases associated with inflammation such as asthma (Rhen and Cidlowski 2005). However, the majority of COPD patients show resistance to even high doses of inhaled glucocorticosteroids, indicating steroid resistance of the underlying inflammatory response (Barnes 2013). Inhaled glucocorticosteroids appear only to be effective in a subgroup of COPD patients with frequent exacerbations (GOLD 2015). Oxidative stress is an important cause of glucocorticosteroids resistance in COPD, as ROS can reduce the activity of histone deacetylase, therefore leading to imbalance of acetylation-deacetylation states of histones (Adenuga and Rahman 2007; Marwick et al. 2007; Barnes 2013).

Airway smooth muscle in COPD

ASM plays a critical role in obstructive pulmonary diseases such as COPD, and is therefore believed to represent a suitable therapeutic target. Indeed, the most effective pharmacological strategy to improve the FEV_1 of COPD patients is represented by a change in ASM tone by bronchodilators, such as β_2-agonists and anticholinergics (GOLD 2015). The contraction of ASM is mediated by G-protein coupled receptors (GPCRs) (Billington and Penn 2003). GPCRs are the largest family of cell surface receptors, structurally characterized by seven transmembrane peptide chains (Kobilka 2007). Agonist binding induces a conformational change and subsequently dissociation of the G_α subunit from the $G_{\beta\gamma}$ dimer of the G-protein, and modulation of downstream effectors depend on the type of G_α subunit: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$ or $G_{\alpha 12/13}$ (Kobilka 2007). In ASM cells, activation of bronchodilating GPCRs that couple to $G_{\alpha s}$, such as β_2-adrenoreceptors, leads to an increase of intracellular cAMP and activation of PKA, thereby causing an inhibitory phosphorylation of myosin light chain (MLC) kinase and a subsequent reduction in myosin phosphorylation. This will eventually reduce the interaction between actin and myosin filaments and results in bronchodilation (Montuschi et al. 2014). Recent studies demonstrate that Epac also reduces MLC phosphorylation, a process involving the small GTPases Rac, and induces relaxation of pre-contracted ASM (Roscioni et al. 2011b). In contrast, activation of bronchoconstrictive GPCRs that couple to $G_{\alpha q}$, such as histamine H1 receptors or muscarinic acetylcholine (M_3) receptors, cause an increase in intracellular calcium and thereby increase myosin phosphorylation, which triggers ASM contraction and bronchoconstriction (Montuschi et al. 2014).

Bronchodilators are widely used in the treatment of airflow limitation in COPD, reducing airflow obstruction and dynamic hyperinflation, and improving exercise performance (GOLD 2015). By reducing airway smooth muscle (ASM) tone, bronchodilators can significantly improve FEV_1 in COPD patients (GOLD 2015). Currently, inhaled β_2-adrenoreceptor agonists (β_2-agonists) and anticholinergics, as well as combinations thereof, are commonly used as bronchodilators that provide effective symptomatic relief (Cazzola et al. 2011). While anticholinergics relax ASM by antagonism of M_3 muscarinic receptors (a bronchoconstrictive

GPCR), β_2-agonists exert their function upon interaction with and activation of the β_2-adrenoreceptor (a bronchodilating GPCR). In the present thesis, we mainly focus on β_2-agonists. β_2-Agonists exert their function upon interaction with and activation of the β_2-adrenoreceptor(Nelson 1995). β_2-Agonists activate $G_{\alpha s}$-proteins that subsequently activate adenylyl cyclases, which catalyze the conversion of adenosine triphosphate (ATP) into bioactive cyclic adenosine monophosphate (cAMP). The second messenger cAMP in turn activates protein kinase A (PKA), exchange protein directly activated by cAMP (Epac) and certain ion channels (Grandoch et al. 2010; Dekkers et al. 2013; Schmidt et al. 2013). cAMP signaling is terminated by phosphodiesterase (PDEs), which degrade cAMP into inactive 5'AMP (Taskén and Aandahl 2004). Several intracellular proteins are phosphorylated by the cAMP-dependent PKA, leading to several therapeutic effects including the relaxation of ASM (Billington et al. 2013) (see also below), increased mucociliary clearance (Hasani et al. 2005), antagonism of pro-inflammatory transcription factors (such as NF-κB) (Oldenburger et al. 2012) and induction of synthesis of the anti-inflammatory cytokines (such as interleukin (IL)-10) (Theron et al. 2013).

Although β_2-agonists, next to anticholinergics, are widely used in COPD patients as bronchodilators, they possess several undesirable side effects. Repeated and prolonged treatment of β_2-agonists can cause desensitization of the β_2-adrenoceptor, which is believed to be one of the main mechanisms of β_2-agonists tolerance (Broadley 2006). Another side effects of β_2-agonists is caused by activation of β-adrenoceptors in extrapulmonary peripheral organs, leading to muscular tremor or tachycardia (Broadley 2006; Montuschi et al. 2014). Therefore, for patients suffering cardiovascular diseases, β_2-agonists always need to be administered with caution (Montuschi et al. 2014).

Airway epithelium in COPD

The airway epithelium is a tight structural cell barrier that acts as a first line of defense against inhaled foreign materials. However, exposure to cigarette smoke reduces the epithelial barrier function (Oldenburger et al. 2014). In addition, airway epithelial cells are also actively involved in the development of COPD. Airway epithelial cells are one of the major sources of inflammatory and fibrotic mediators, such as tumor necrosis factor α (TNF-α), transforming growth factor-β (TGF-β), IL-8 and matrix metalloproteinases (MMPs), therefore contributing to small airways inflammation and fibrosis (Mio et al. 1997; Takizawa et al. 2001; Hellermann et al. 2002; Barnes 2009). Goblet cells, a special type of epithelial cells, produce excessive mucus in the presence of cigarette smoke, and are thereby believed to contribute to airflow limitation in COPD (Shao et al. 2004). Moreover, cigarette smoke induces cycles of injury and repair in the epithelium, leading to increase in epithelial permeability and a loss of epithelial barrier properties, which is another important feature of COPD (Puchelle et al. 2006; Olivera et al. 2007; Heijink et al. 2012).

Inflammatory cells in COPD

As mentioned before, the pathophysiology of COPD involves several types of inflammatory cells, including macrophages, neutrophils and T-lymphocytes known to produce multiple inflammatory mediators (Barnes et al. 2003; Barnes 2004). Macrophages play a pivotal role in the pathophysiology of COPD (Barnes 2004). Thus, exposure to cigarette smoke or other inhaled irritants activates macrophages that orchestrate the inflammatory process in COPD upon secretion of IL-8, leukotriene (LT) B_4 and proteolytic enzymes (especially MMP-9, MMP-12) (Angelis et al. 2014). IL-8 and LTB_4 attract neutrophils to the respiratory tract and lead to further airway inflammation (Barnes 2004). Release of MMPs causes elastolysis and thus contributes to the development of pulmonary emphysema (Barnes 2004).

Neutrophilic inflammation is a prominent feature of COPD. Neutrophils are recognized as a first line of defense against microbial invasion (Amulic et al. 2012) and the most abundant inflammatory cell type present in the airways and lung parenchyma of COPD patients (Pesci et al. 1998; Pilette et al. 2007). Cigarette smoke and other pathogens trigger the release of IL-8 from various cells as mentioned above, promoting the recruitment of neutrophils. Degranulation of neutrophils leads to the release of mediators including neutrophil elastase and MMP-9, both implicated in tissue damage and emphysema in COPD (Owen 2008). Inhaled irritants (such as cigarette smoke and air pollutants) create further pro-inflammatory signals by inducing secondary necrosis through impairing the removal of apoptotic neutrophils (Hoenderdos and Condliffe 2013). Moreover, in addition to macrophages, neutrophils are one of the main sources of ROS produced during the development and progression of COPD (Noguera et al. 2001; Vaitkus et al. 2013).

Besides macrophages and neutrophils, the lung parenchyma and airways of COPD patients are characterized by increased infiltration of T-lymphocytes (CD8+ and CD4+) (Saetta et al. 1998; Retamales et al. 2001), which correlates with the development of emphysema (Finkelstein et al. 1995; Majo et al. 2001) and the severity of airflow obstruction (O'Shaughnessy et al. 1997; Saetta et al. 1999). Although the mechanism of T-lymphocyte accumulation in the lungs of COPD patients is not fully understood, one study has indicated that T-lymphocytes of COPD patients have increased expression of the chemokine receptor CXCR3 (Saetta et al. 2002), thereby allowing the chemotaxis of T lymphocytes via CXCR3-activating chemokines, such as CXCL10, which are released by bronchiolar epithelial cells (Spurrell et al. 2005).

Cellular Processes Involved in COPD

Cigarette smoke

Since its identification as a risk factor about 40 years ago (Fletcher et al. 1977), tobacco smoke has been established as the most commonly encountered risk factor for the development

of COPD (GOLD 2015). Smoking cessation is the most effective intervention to influence the progression of COPD and to slow down the exaggerated decline in lung function. It has been reported that non-smokers experience a smaller prevalence of respiratory symptoms and lung function abnormalities, a lower annual rate of FEV_1 decline, and a lower COPD rate than cigarette smokers (Kohansal et al. 2009).

Cigarette smoke contains about 5300 identified components, including nicotine, acrolein, formaldehyde and acetaldehyde as well as lipopolysaccharide (LPS) (Rodgman and Perfetti 2013), which can actively participate in the process of airway inflammation via the release of (pro-)inflammatory cytokines and mucus hypersecretion (Borchers et al. 1999; de Jonge and Ulloa 2007; Facchinetti et al. 2007; Haswell et al. 2010; Lee et al. 2012).

Next to the above mentioned chemical components, cigarette smoke is also a rich source of ROS that are responsible for several types of airway damage induced by oxidative stress (Church and Pryor 1985; Barnes et al. 2003; Domej et al. 2014). Cigarette smoke is reported to induce the intracellular level of ROS in various lung cells such as epithelial cells (Tang et al. 2011) and ASM cells (Wylam et al. 2015). The increased levels of ROS and oxidative stress in the cells subsequently activate several pro-inflammatory transcriptional factors, such as nuclear factor (NF)-κB, and eventually results in the production of various inflammatory mediators including IL-8 (Evans et al. 2003; Kirkham and Barnes 2013). Indeed, treatment with cigarette smoke or cigarette smoke extract (CSE) dose-dependently increases the release of IL-8 from cultured ASM cells by increasing NF-κB activity (Tang et al. 2011; Oldenburger et al. 2012; Pera et al. 2012; Chen et al. 2014).

Oxidative stress

Oxidative stress, which is a result from an anti-oxidant/oxidant imbalance, is the physiological damage that occurs in the presence of ROS (Domej et al. 2014). Compared to other organs, ROS seem to exert profound effects in the lung due to its direct and constant exposure to an environment with a higher oxygen load (Kinnula and Crapo 2003). Consequently, the lung has sophisticated defense mechanisms against oxidative stress, including nuclear factor erythroid 2-related factor 2 (Nrf2), the "master regulator" of antioxidant response (Comhair and Erzurum 2010). Nrf2 is a nuclear factor that controls anti-oxidative responses of cells (Itoh et al. 1997). During the resting state, the activity of Nrf2 is suppressed by binding to Keap1, preventing its nuclear translocation. Under oxidative stress, Keap1 dissociates from Nrf2, thereby allowing its nuclear translocation (Kobayashi et al. 2004). Subsequently, Nrf2-induced anti-oxidative factors start to alleviate oxidative stress responses (Itoh et al. 1999) (Figure 1).

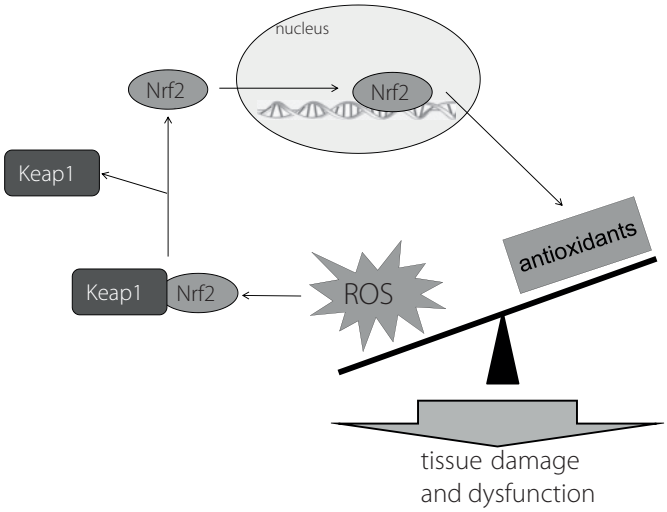

Figure 1. Nrf2 activation. During the resting state, the activity of Nrf2 is suppressed by Keap1 upon prevention of its nuclear translocation. Imbalance between ROS and anti-oxidants induces tissue damage and dysfunction. Under oxidative stress, Keap1 dissociates from Nrf2 thereby allowing its nuclear translocation. Subsequently, Nrf2-induced anti-oxidative elements start to alleviate oxidative stress responses.

Currently, researchers put more and more focus on oxidative stress, as it is believed to play an important and central role in the pathogenesis of COPD (Barnes 2004; Kirkham and Barnes 2013) (Figure 2). COPD patients fail to maintain the anti-oxidant/oxidant balance due to either the dysfunction of those anti-oxidative stress mechanisms or due to inhalation of excessive ROS (e.g. via cigarette smoke) (Barnes 2004; Kirkham and Barnes 2013). ROS derived from environmental pollution, cigarette smoke and/or inflammatory cells, such as neutrophils, cause an increase in pulmonary oxidative stress and thereby contribute to the development and progression of COPD (Barnes 2004; Rahman and Adcock 2006; van Eeden and Sin 2013; Domej et al. 2014) (Figure 2). Moreover, studies have shown that cigarette smoke can cause mitochondrial dysfunction, thereby inducing ROS production in ASM cells (Aravamudan et al. 2014; Wiegman et al. 2015) and epithelial cells (van der Toorn et al. 2009). Excessive pulmonary ROS production leads to the activation of the pro-inflammatory transcription factors, such as NF-κB and activator protein-1 (AP-1) (Morgan and Liu 2011). Subsequently, the activated pro-inflammatory transcription factors lead to the up-regulation of adhesion molecules and increased release of pro-inflammatory mediators such as IL-6, IL-8, TNF-α (Mittal et al. 2014). Activation of the NF-κB can be activated by excessive ROS production in airway epithelial (Li et al. 2013) and ASM cells (Luo et al. 2009), thereby resulting in an increased secretion of IL-8 (Oenema et al. 2010; Oldenburger et al. 2012; Pera et al. 2012).

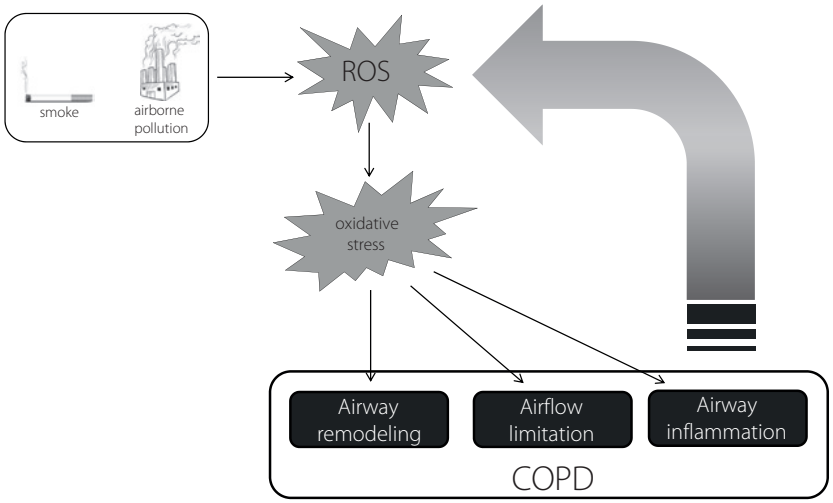

Figure 2. Oxidative stress in COPD. ROS derived from cigarette smoke and environmental pollution cause an increase in pulmonary oxidative stress and thereby contribute to the development and progression of COPD, leading to airway inflammation, airflow limitation and remodeling. Inflammatory cells such as neutrophils, produce excessive ROS, therefore further increasing the oxidative stress.

Oxidative stress is also implicated in fibrosis in a variety of organs, including the lungs (Cheresh et al. 2013). TGF-β is the most potent and ubiquitous pro-fibrogenic cytokine and its expression is increased in COPD (Liebhart and Dobek 2009; Mak et al. 2009). Evidence indicates that ROS play a key role in TGF-β-induced fibrosis by activating ASM cells, endothelial cells, airway epithelial cells and fibroblasts (Liu and Gaston Pravia 2010). While TGF-β is able to increase intracellular ROS production, ROS can also activate latent TGF-β and induce TGF-β gene expression, thereby creating a vicious circle (Koli et al. 2008; Liu and Gaston Pravia 2010). In support of a pro-fibrotic role for ROS, studies showed that exogenous antioxidants could attenuate the development of fibrosis (Liu and Gaston Pravia 2010), suggesting the possibility of using an anti-oxidant compound as a novel treatment for fibrosis.

Besides inflammation and fibrosis, ROS can also trigger the peroxidative breakdown of lipids, a process implicated in increased airway epithelial permeability (Bromberg et al. 1991; Boardman et al. 2004; Rahman 2005; Rahman and Adcock 2006; Shintani et al. 2015). The major product of the lipid oxidation reaction, malondialdehyde (MDA), is also one of the most reactive electrophile species that causes further toxic stress to the cells (Farmer and Davoine 2007; Ayala et al. 2014). Moreover, studies indicate that oxidative stress is an important cause of glucocorticosteroid resistance in COPD, as ROS alter histone deacetylase functioning, causing reduced suppression of pro-inflammatory genes by corticosteroids, including NF- κB (Adenuga and Rahman 2007; Marwick et al. 2007; Barnes 2013). Last but not

least, there is evidence suggesting that oxidative stress directly increase ASM contractility, which might contribute to the symptoms of COPD (Hulsmann et al. 1994; Rahman 2009; Berair et al. 2013).

Hydrogen sulfide

In addition to the gaseous neurotransmitter nitric oxide and carbon monoxide, hydrogen sulfide (H_2S) has been added to the list of important gaseous biological mediators. H_2S is synthesized in the cytoplasm from L-cysteine, a process involving several enzymes such as cystathionine-β synthetase (CBS), cystathionine-γ lyase, cysteine aminotransferase, 3-mercaptopyruvate sulphurtransferase and cysteine lyase (Whiteman and Moore 2009; Li et al. 2011).

Exogenous H_2S seems to exert anti-inflammatory effects by downregulating pro-inflammatory cytokines, including IL-8, IL-6 and IL-1 β, and upregulating anti-inflammatory cytokines, such as IL-10, in rodent models of lung injury, induced by either tobacco smoke (Esechie et al. 2008) or oleic acid (Li et al. 2008). Besides anti-inflammatory properties, exogenous H_2S also showed bronchodilatory effects by reducing AHR induced by tobacco smoke in rodent models (Chen et al. 2009; Chen et al. 2011; Han et al. 2011).

Some argue that the therapeutic effects of H_2S is (at least partly) attributed to its anti-oxidative capacities (Chen et al. 2008; Esechie et al. 2008; Whiteman et al. 2010; Perry et al. 2011; Faller et al. 2012; Han et al. 2015b). In support, the anti-inflammatory and bronchodilatory effects of H_2S are associated with anti-oxidative properties, measured by increased Nrf2 expression and glutathione/oxidized glutathione ratio in the lungs (Han et al. 2011). In accordance, H_2S was reported to activate Nrf2 by inhibition of Keap1 via formation of the C226–C613 disulfide bond on Keap1 protein in mouse embryonic fibroblasts (Hourihan et al. 2013). In contrast, H_2S possesses a high redox potential that may provoke pro-inflammatory responses. Studies showed that oxidative stress originating from activated neutrophils converts H_2S to sulfite (Mitsuhashi et al. 2005), which is considered an inflammatory mediator in airway diseases (Ratthé et al. 2002; Mitsuhashi et al. 2004). These findings may explain the fact that H_2S was also found to induce the secretion of pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNF-α) in other studies (Zhi et al. 2007; Bhatia et al. 2008). Currently, the usage of H_2S or its donors as potential treatments for COPD is still under debate.

LPS-induced Guinea Pig Models of COPD

For ethical reasons and limited availability of human tissue, animal models are frequently used to study the pathogenesis of COPD. COPD is closely related to the structure of the lung, therefore a good animal model should have pulmonary structure similar to that of humans (Wright et al. 2008). While none of present animal models can perfectly reproduce

the pathophysiology of human COPD, rats, mice and guinea pigs are able to mimic specific symptoms of the disease, including infiltration of inflammatory cells such as neutrophils, cytokine production, emphysema, airway remodeling and the development of AHR (Wright et al. 2008; Han et al. 2016; Oliveira et al. 2016). As we mentioned above, cigarette smoke is the major risk factor for the development of COPD (GOLD 2015), and is therefore used in many COPD studies (Wright et al. 2008). LPS is a component of the gram-negative bacterial cell wall (Kulp and Kuehn 2010), and represents an active component in cigarette smoke (Hasday et al. 1999). The complex mechanisms underlying the LPS-induced inflammation in the lung are not fully studied. In guinea pigs and rats, several studies have shown that LPS induces COPD-like symptoms, including AHR, inflammation and airway remodeling, by binding to cell surface pattern recognition receptors encompassing toll-like receptor 4, CD14, LPS-binding protein, and myeloid differentiation-2 (Brass et al. 2003; Brass et al. 2004; Savov et al. 2005; Brass et al. 2007; Pace et al. 2015). Upon activation of those receptors, LPS induces the production of pro-inflammatory cytokines such as IL-8 in the airways, which leads to the influx and activation of inflammatory cells such as neutrophils (Fricker et al. 2014).

Although most current studies are using mice or rats as animal model for COPD, in this thesis we used a guinea pig model. Guinea pigs have also been used as small animal species in preclinical studies related to asthma and COPD (Canning 2003). In contrast to mice and rats, pulmonary biological mediators and anatomy in guinea pigs are quite similar to that of humans (Canning 2003; Canning and Chou 2008), leading to the fact that experimental interventions in the lung are comparable (Canning 2003; Canning and Chou 2008). Guinea pigs give a fast inflammatory reaction upon exposure of LPS. There was a significant influx of neutrophils, along with other inflammatory cells, into the airways 24 hours after exposure of LPS (Toward and Broadley 2000; Toward et al. 2004; Smit et al. 2013). Although often underestimated in clinical assessment, AHR is an important characteristic of COPD (van den Berge et al. 2012) that associates with accelerated lung function decline (Postma et al. 1986; Tashkin et al. 1996). There is a strong association between AHR and accumulation of lung neutrophils (van den Berge et al. 2012). Similar to those clinical findings, exposing guinea pigs with LPS acutely or chronically has been shown to induce significant AHR (Toward and Broadley 2000; Toward et al. 2004; Kaneko et al. 2007; Smit et al. 2013). Intranasally applied or inhaled LPS mimicked pathologic and inflammatory changes in guinea pigs that are also observed in human subjects with COPD and LPS-treated guinea pig are therefore used as *in vivo* models for COPD (Toward and Broadley 2000; Toward and Broadley 2002; Toward et al. 2004; Kaneko et al. 2007; Pera et al. 2011; Baarsma et al. 2013; Smit et al. 2013; Pera et al. 2014). In this thesis, we used as an acute guinea pig model of COPD using a single exposure to LPS. Earlier studies from our group as well as of others have shown that the main characteristics of this model are infiltration of neutrophils (Toward and Broadley 2000;

Smit et al. 2013), a feature that correlates strongly with clinical outcomes (Stockley 2006; Gernez et al. 2010; Hoenderdos and Condliffe 2013), and AHR (Toward and Broadley 2000; Smit et al. 2013).

Novel Therapeutic Avenues for COPD

Sul compounds

As we mentioned above, the COPD is characterized by persistent and progressive airflow limitation and featured with an enhanced chronic airway inflammation (GOLD 2015). However, the current medications are not always effective and fail to reduce the progression of COPD (Barnes 2013). Therefore, studies for novel medicines are necessary. Recently, a series of novel compounds (named as Sul compounds) were synthesized. They have shown to protect cells form oxidative stress induced by hypothermia and rewarming (Patent: WO2014098586 A1, Van et al. 2014). Moreover, preliminary studies suggested that Sul compounds might activate cystathionine β synthase, a main enzyme for cellular endogenous H_2S production (Dugbartey et al. 2014). As both the anti-oxidative property and H_2S would beneficially affect the pathophysiology of COPD, these Sul compounds may represent potential novel treatments of obstructive lung disorders such as COPD.

A-kinase anchoring proteins

Compartmentalization of cellular signaling is a common strategy used to ensure the accuracy and efficiency of cellular responses (McCormick and Baillie 2014). Over the past years, it is generally accepted that spatial-temporal mechanisms regulate cAMP pools within individual cells. Several studies indicated that the communication between β_2-adrenoreceptor, cAMP effectors, PDEs and other downstream targets are coordinated by A-kinase anchoring proteins (AKAPs) (Figure 3) (Logue and Scott 2010; Dekkers et al. 2013). AKAPs are a group of scaffolding proteins with the ability to associate with PKA via a short α-helical structure. Members of the AKAP superfamily act as targeting devices that assemble a large variety of structural and signaling molecules and thereby help those signaling elements to localize to different cellular microdomains (Scott and Santana 2010; Logue and Scott 2010). Recently our group was the first to publish that dysfunction of AKAP complexes contributes to features of COPD (Oldenburger et al. 2014; Poppinga et al. 2015). Thus, disruption of the interaction between AKAPs and PKA further increases inflammatory processes and loss of epithelial barrier function induced by cigarette smoke (Oldenburger et al. 2014; Poppinga et al. 2015). In addition, the expression of several AKAPs, including AKAP5 and AKAP12, is lower in lung tissue of COPD patients and in ASM cells exposed to cigarette smoke. Each AKAP is featured with a amphipathic helix domain responsible for PKA RII subunit binding (Carr et al. 1992). St-Ht31 is a stearated form of a peptide containing

the amphipathic helix domain (human thyroid AKAP), which allows it to competitively inhibit the interaction of PKA RII subunits with AKAPs (Carr et al. 1992) (Figure 3). Currently, st-Ht31 is used to study the role of PKA-AKAP complexes in several biological processes, including in the lung (Poppinga et al. 2014; Han et al. 2015a). The following section briefly describes the AKAP proteins in airway epithelial cells and ASM cells.

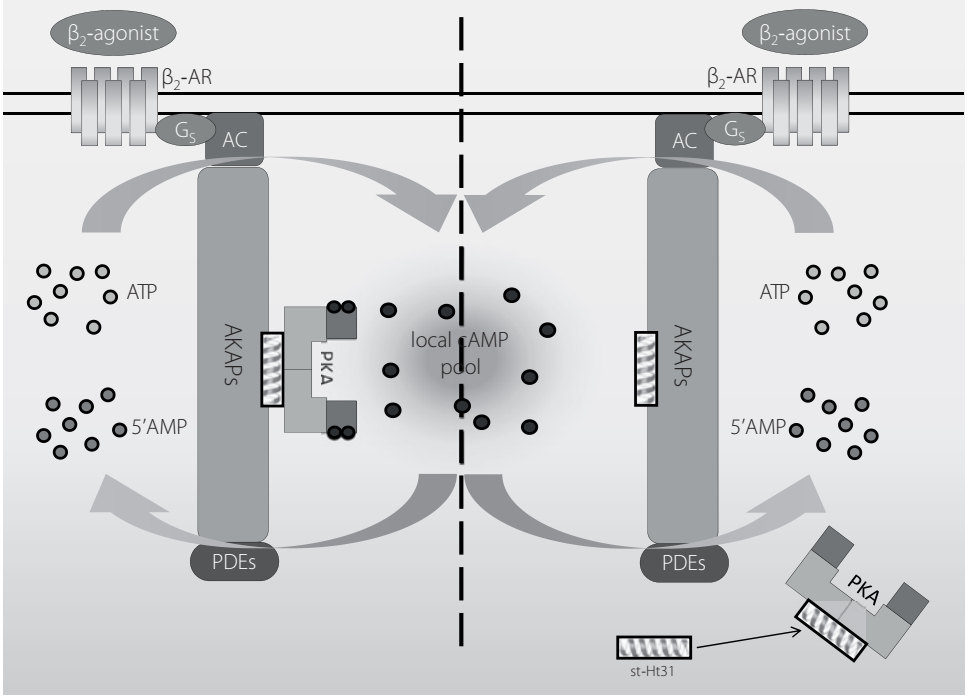

Figure 3. Compartmentalization of cAMP signaling by A-kinase anchoring proteins (AKAPs). After activation of β2-adrenoceptors by their appropriate ligands, adenylyl cyclase (AC) is stimulated leading to the generation of cyclic adenosine monophosphate (cAMP) and activation of cAMP effectors, such as protein kinase A (PKA). Phosphodiesterases (PDEs) decrease the cellular cAMP level by cleavage to 5'AMP. By binding PKA, PDEs, AC and other proteins, AKAPs help to form localized cAMP pools. AKAPs organize the anchoring of PKA in the vicinity of such cAMP pools, thus controlling its maintenance in space and time. All AKAPs are featured with a amphipathic helix domain responsible for PKA RII subunit binding. The synthetic peptide st-Ht31 mimics this amphipathic helix domain (human thyroid AKAP) and thereby competitively inhibits the interaction of PKA RII subunits with other AKAPs.

In 2001, Huang and coworkers suggested that the compartmentalization of cAMP signaling at the inner apical membrane surface allows cells to selectively activate the apical functions needed for the protective response to physical luminal stimuli (Huang et al. 2001). Later on, Barnes and coworkers identified another example of compartmentalization, involving

phosphodiesterases (PDE)4D, the main PDE expressed in the lungs, which limit cAMP effects to short distances from the apical membrane by subcellular localization of PKA (Barnes et al. 2005). Recently, a study indicated that anchoring of PKA to AKAPs is critical for cAMP modulation of the cystic fibrosis transmembrane conductance regulator (CFTR) in airway epithelial cells (Monterisi et al. 2012). Indeed, our previous studies also demonstrate that AKAP5, AKAP9 and AKAP12 are expressed in human bronchial epithelial (HBE) cells, and that exposure of HBE cells to CSE resulted in an alteration of AKAP expression accompanied with a loss of barrier function (Oldenburger et al. 2014). Importantly, disruption of AKAP-PKA interactions by st-Ht31 prevented the CSE-induced reduction of E-cadherin and AKAP9 protein expression and subsequent loss of barrier function, suggesting that AKAP complexes contribute to the maintenance of epithelial barrier.

As ASM also expresses several AKAPs (e.g. AKAP5) (Horvat et al. 2012; Poppinga et al. 2015), recruitment of PDE and PKA to a distinct subset of AKAPs might bear the potential to control cellular cAMP pools in ASM as well. The importance of the cellular cAMP homeostasis in ASM has been shown in studies on the "β-agonist paradox". Overexpression of the β_2-adrenoceptor (McGraw et al. 2003) or adenylate cyclase (AC) subtype 5 (Wang et al. 2011) leads to AHR, instead of the anticipated reduction of both as both are involved in relaxing ASM. Due to these studies and several others it has become clear that the intracellular cAMP gradient in human ASM is spatially controlled by PDEs (Billington and Hall 2012). Although representing a minor component of the lung PDE pool, it has been shown that inhibition of PDE4D5 by rolipram augmented isoproterenol-induced cAMP production, suggesting that this PDE subtype plays a key role as physiological regulator of β_2-adrenoreceptor-induced cAMP elevations (Billington et al. 2008). Furthermore, research has shown that PDE4D deficient mice neither respond to cholinergic stimulation nor develop AHR after exposure to antigen, presumably due to their inability to decrease-cellular cAMP (Hansen et al. 2000). Due to the intrinsic property of plasticity, ASM cells not only contract but also have the ability to produce inflammatory mediators (Halayko and Amrani 2003; Wright et al. 2013). It has been shown that cAMP-mobilizing agents, including β-agonists, reduce CSE-induced IL-8 release in ASM cells (Oldenburger et al. 2012). Thus, subtle alterations in spatio-temporal regulation of cellular cAMP might profoundly change diverse cellular responses of ASM beyond relaxation. It is tempting to speculate that AKAP might contribute to the distinct functional responses of ASM. Recent studies demonstrate that disruption of AKAP-PKA complexes in ASM changed only cAMP elevations close to the membrane, leaving global cAMP production unaffected (Horvat et al. 2012), as well augments CSE-induced IL-8 release, a process involving signaling to extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Poppinga et al. 2015).

Besides inducing ASM relaxation and inhibiting cytokine release from ASM cells, activation of the cAMP effectors PKA and Epac also inhibit growth factor-induced DNA synthesis in

ASM cells, a process involving ERK1/2 and p70S6 kinase (Roscioni et al. 2011a; Roscioni et al. 2011b). Activation of PKA and Epac also prevents the development of the growth factor-induced hypocontractile ASM phenotype (Roscioni et al. 2011a; Roscioni et al. 2011b), demonstrating that cAMP signaling also regulates phenotypic plasticity of ASM cells. The involvement of PKA-AKAP complexes have not yet been studied in the regulation of ASM cell plasticity. However, studies indicate that PKA-AKAP complexes regulate processes that are important for the regulation of the cell cycle in ASM (Collas et al. 1999; Indolfi et al. 2001; Landsverk et al. 2001; Alfthan et al. 2004; Laulajainen et al. 2008; Smith et al. 2010; Gao et al. 2012). Therefore, AKAP-PKA interactions may be important in regulating processes as ASM tone, ASM proliferation and cytokine release from ASM and are thus interesting targets for novel therapies of COPD.

Scope of the Thesis

The objective of this thesis is to investigate possible novel targets for the treatment of COPD. Special focus lies on the involvement of PKA-AKAP complexes and oxidative stress. Using *in vitro*, *ex vivo* and *in vivo* approaches, we explored the therapeutic potential and underlying molecular mechanisms of several novel compounds (Sul compounds) for the treatment of COPD. The contribution of PKA-AKAP complexes to ASM responses have been studied by using the peptide st-Ht31, known to interfere with the binding of PKA to AKAP proteins.

In Chapter 2, we summarize the potential of novel targets and tools to study these, including anti-inflammatory drugs, inhibitors of PDEs as well as kinase inhibitors for the treatment of asthma and COPD. We discussed the potential role of PKA-AKAP complexes in modulating the effect of drugs used to treat obstructive lung diseases, and we suggest that AKAPs may be a novel therapeutic target acting downstream of GPCRs to treat COPD.

Since ASM plays a critical role in obstructive pulmonary diseases including COPD, ASM therefore represents a very suitable therapeutic target. ASM can contribute to the progress of airflow limitation by inducing increased airway narrowing, which may involve remodeling of the ASM characterized by hyperplasia and hypertrophy (Chung 2005). β_2-Agonists are important bronchodilators via elevation of cellular cAMP levels and subsequent activation of the downstream effectors PKA and Epac (Cazzola et al. 2011; Oldenburger et al. 2012). AKAPs coordinate responses driven by either PKA and/or Epac (Grandoch et al. 2010), and have recently been shown to be expressed in the airways (Horvat et al. 2012; Poppinga et al. 2015). Using st-Ht31, we studied the role of the PKA-AKAP complexes in phenotypic plasticity of ASM. (Chapter 3). The effect of st-Ht31 on markers of the proliferative ASM phenotype, such as DNA synthesis and activation of cell cycle proteins, was studied in cultured human ASM cells. Since AKAP8 is known to interact with cell cycle proteins such as cyclin D1, we also studied the (sub)cellular expression of AKAP8 in ASM in the absence and

presence of st-Ht31. In addition, the effect of st-Ht31 on the expression of the contractile proteins α-smooth muscle actin and calponin, markers for the contractile ASM phenotype, was studied in ASM cells. The expression of α-smooth muscle actin and the proliferative marker PCNA (proliferating cell nuclear antigen) were also studied in intact human tracheal smooth muscle strips following overnight treatment with st-Ht31. Lastly, the effect of this overnight treatment with st-Ht31 on methacholine-induced contractility was studied in the human tracheal smooth muscle strips.

A series of highly sophisticated, strictly regulated molecular mechanisms are required to maintain the cell cycle progression, including cyclins and Rb (Bertoli et al. 2013; Dick and Rubin 2013; Zitouni et al. 2014). Dysfunction of this process could lead to the development of diseases like cancer as well as COPD (Sundar et al. 2011; Sperka et al. 2012). Cellular signaling compartmentalization is considered a common strategy to ensure the accuracy and efficiency of cellular signaling in cell cycle regulation (McCormick and Baillie 2014). In Chapter 4, we reviewed the recent knowledge about the role of AKAPs in the regulation and progression of the cell cycle in a pathophysiological context.

Although many COPD patients are treated with anti-inflammatory corticosteroids, most patients respond poorly to these drugs due to low sensitivity, urging better anti-inflammatory treatment (Barnes et al. 2002; Broadley 2006). A series of novel compounds (Sul-90, Sul-121, Sul-127 and Sul-136) have shown promising cell protective effects from oxidative stress (Van der Graaf et al. 2014). In Chapter 5, we screened these compounds based on their pharmacological potential as novel treatment options for COPD, with a special focus on their potential anti-inflammatory effects on cultured ASM cells and relaxing properties on pre-contracted ASM strips. We used the most promising compound (Sul-121) in Chapter 6 to explore its protective role on neutrophilia and AHR in an LPS-induced guinea pig model of COPD. Using a combination of *in vivo* and *in vitro* studies, we studied the molecular mechanisms involved in the effects of Sul-121 on LPS and CSE-induced inflammatory processes. The effect of Sul-121 on CSE-induced IL-8 release, ROS production, H_2S levels, and the activation of the anti-oxidative response factor Nrf2 was evaluated.

All results and findings are summarized and discussed in Chapter 7 and a future perspective in relation to the treatment of obstructive lung disorders is given.

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